

1 **Title Page**

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4 **α_1 -antitrypsin polymerizes in alveolar macrophages of smokers with and without**
5 **α_1 -antitrypsin deficiency.**

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26

27 **Running Title:** AAT Polymerization in alveolar macrophages

28

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30

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34

35 **Abbreviations:** AAT= α_1 -antitrypsin; AM= Alveolar Macrophages; AATD= α_1 -antitrypsin
36 deficiency; COPD = Chronic Obstructive Pulmonary Disease; Glu= glutamic acid; Lys= lysine;
37 ER= endoplasmic reticulum; NF- κ B= nuclear factor-kappaB; GM-CSF= Granulocyte-macrophage
38 colony-stimulating factor; LPS= Lipopolysaccharides; IL= Interleukin; PAS= periodic acid-Schiff;
39 HPF= high-power fields; BAL= bronchoalveolar lavage;

40

41 **ABSTRACT**

42 **Background.** The deficiency of α_1 -antitrypsin (AAT) is secondary to misfolding and
43 polymerization of the abnormal Z-AAT in liver cells and is associated with lung emphysema.
44 Alveolar macrophages (AM) produce AAT, however it is not known if Z-AAT can polymerize in
45 AM, further decreasing lung AAT and promoting lung inflammation.

46 **Aims.** To investigate if AAT polymerizes in AM and to study the possible relation between
47 polymerization and degree of lung inflammation.

48 **Methods.** Immunohistochemical analysis with 2C1 monoclonal antibody specific for polymerized
49 AAT was performed in sections of: 9 lungs from individuals with AAT deficiency (AATD) and
50 severe COPD, 35 smokers with normal AAT levels of which 24 with severe COPD and 11 without
51 COPD, and 13 non-smokers. AM positive for AAT polymers were counted and expressed as
52 percentage of total AM in lung.

53 **Results.** AAT polymerization was detected in [27(4-67)%] of AM from individuals with AATD but
54 also in AM from smokers with normal AAT with [24(0-70)%] and without [24(0-60)%] COPD, but
55 not in AM from non-smokers [0(0-1.5)%] ($p < 0.0001$). The percentage of AM with polymerized
56 AAT correlated with pack-years smoked ($r = 0.53$, $p = 0.0001$), FEV₁/FVC ($r = -0.41$, $p = 0.005$),
57 number of CD8+T-cells and neutrophils in alveolar walls ($r = 0.51$, $p = 0.002$; $r = 0.31$, $p = 0.05$
58 respectively).

59 **Conclusions.** Polymerization of AAT in alveolar macrophages occurs in the lungs of individuals
60 with AATD but also in smokers with normal AAT levels with or without COPD. Our findings
61 highlight the similarities in the pathophysiology of COPD in individuals with and without AATD,
62 adding a potentially important step to the mechanism of COPD.

63

64 **Key words:** COPD, emphysema, serpins

65

66 **INTRODUCTION**

67 α_1 -antitrypsin (AAT) is the archetypal member of the serine protease inhibitor (SERPIN)
68 superfamily. Severe deficiency of this protein, secondary to an inherited disorder, is linked to the
69 development of early onset emphysema. About 95% of the significant clinical deficiency is caused
70 by the Z variant of the protein that results from the substitution of a glutamic acid (Glu) by a lysine
71 (Lys) at position 342.¹⁻⁵ Approximately 0.06% of individuals of North European descent have
72 severe deficiency of AAT with plasma levels of less than 0.2 g/L.¹⁻⁵ The Glu to Lys substitution in
73 Z-AAT results in abnormal protein folding within the endoplasmic reticulum (ER) of the
74 hepatocyte, protein polymerization and intracellular retention with consequent low AAT serum
75 levels.¹⁻⁵ Thus the effect of the Z mutation is not a failure of synthesis (Z-AAT is processed
76 normally until it reaches the final stage of the hepatocyte ER pathway), but a failure in folding and
77 secretion. About 85% of the Z-AAT is removed by ER-associated degradation or aggregates to
78 form polymers while 15% is secreted in the serum.¹⁻⁶

79 Polymerization of Z-AAT in the liver causes a “toxic gain of function” within hepatocytes³, with
80 ER stress and activation of NF- κ B⁷⁻⁹ triggering an inflammatory reaction in response to protein
81 misfolding and polymerization in the hepatocytes that predispose to neonatal hepatitis and liver
82 cirrhosis.^{10,11}

83 Epithelial barrier macrophages such as alveolar macrophages, intestinal and epithelial macrophages
84 and breast milk macrophages, along with blood monocytes, are also important producers of AAT in
85 their local milieu.¹²⁻¹⁴ To a minor extent other cells in the lung including lung epithelial cells,
86 bronchial epithelial cells (BECs), endothelial cells, the human A549 cell line of alveolar epithelial
87 cells in the lung and polymorphonuclear leukocytes and neutrophils, have been found to also
88 produce AAT.¹⁵⁻¹⁹ Alveolar macrophages develop from fetal liver under the control of GM-CSF in
89 the first days of life, paralleling the development of the alveoli and then maintain themselves by in
90 situ self-renewal.²⁰⁻²² Perhaps, due to their different origin, there is an important difference in
91 production of AAT between blood monocytes (which produce three fold less AAT) and alveolar
92 macrophages¹⁴, suggesting that alveolar macrophages are preprogramed by their liver origin or that,
93 once in the lung milieu, they up-regulate AAT gene expression.

94 Alveolar macrophages can produce relatively large amounts of AAT directly into the lung but, as
95 with hepatocytes, the production and secretion of AAT is regulated by inflammatory mediators such
96 as Lipopolysaccharides (LPS) and the acute phase cytokine Interleukin IL-6. The synthesis of AAT
97 is also modulated by the presence of elastase in a dose and time dependent way.²³ Under these
98 stimuli wild type PiMM AAT monocytes can increase the synthesis and secretion of AAT by up to
99 10 fold.¹⁴

100 It would seem that the normal production of AAT by alveolar macrophages, potentially increased
101 under the modulation of inflammatory mediators and elastase, could well polymerize in the ER of
102 alveolar macrophages in PiZZ individuals, a possibility that has never been studied in human lung
103 tissue. If that were the case, AAT polymerization in alveolar macrophages will not only contribute
104 to loss of AAT function due to diminished secretion in the alveoli, but also, as in the liver, to “toxic
105 gain of function” with all its complex and detrimental consequences.

106 It was the aim of our study to assess whether alveolar macrophages in the lung tissue from
107 individuals with PiZZ AAT deficiency formed AAT polymers and if polymerization could be
108 related to inflammation within the lung. For this purpose, we studied lung sections from individuals
109 with COPD with AAT deficiency undergoing lung transplantation and compared them with lungs of
110 smokers with COPD and normal AAT (“usual” COPD), smokers without COPD, and non-smokers.
111 The results of this investigation have been presented in abstract form.²⁴

112

113 **METHODS**

114

115 **Subject Characteristics**

116 We studied the tissues from the lungs of 33 patients undergoing lung transplantation for severe
117 COPD: 9 had PiZZ α 1-antitrypsin deficiency (COPD with AATD) and 24 had normal levels of
118 AAT (“usual” COPD). AATD was confirmed by serum levels, together with
119 genotyping/phenotyping in all cases. Sections from the lungs of 11 smokers with normal lung
120 function and 13 non-smoking subjects, who had lung resection for solitary nodules, were included
121 for comparison. All 57 subjects underwent pulmonary function tests prior to surgery and provided
122 informed written consent. The study conformed to the Declaration of Helsinki. All aspects of this
123 study were approved by the local Ethics Committee (reference number 0006045). Details are
124 reported in the Online Supplement.

125 **Histochemistry, immunohistochemistry and morphometric analysis**

126 Lung tissue preparation, histochemistry and immunohistochemistry were performed as previously
127 described and detailed in the Online Supplement.^{25,26}

128 The lung tissue specimens were fixed in formalin, embedded in paraffin wax and cut. At least three
129 lung sections per case were stained with periodic acid-Schiff (PAS) and immunostained according
130 to the standard peroxidase-antiperoxidase method with a commercial polyclonal anti-AAT antibody
131 recognizing total AAT (both native and polymerized, IR505 Dako, Denmark) and with the specific
132 monoclonal antibody 2C1 that recognizes intracellular AAT polymers but not native (monomeric),
133 reactive loop cleaved or latent AAT.²⁷ Negative controls for nonspecific binding were processed
134 either omitting the primary antibody or using isotype IgG and revealed no signal.

135 To quantify AAT positive alveolar macrophages, PAS positive inclusions in alveolar macrophages
136 and AAT polymerized positive alveolar macrophages at least 20 to 40 non consecutive high-power
137 fields (HPF) and at least 100 macrophages inside the alveolar spaces were evaluated for each
138 subject. The results were expressed as percentage of positive macrophages over the total number of
139 macrophages examined.^{25,26} Alveolar macrophages were defined as mononuclear cells with a well-
140 represented cytoplasm, present in the alveolar spaces.

141 As positive control for AAT polymer staining we examined 6 liver samples from PiZZ patients who
142 underwent liver transplantation related to AATD. 5 μ m sections were stained with PAS and the
143 specific monoclonal antibody 2C1 to detect AAT polymerization, following the same protocol used
144 for pulmonary tissue.

145 Neutrophils, macrophages, T CD4+ lymphocytes, T CD8+ lymphocytes and B lymphocytes were
146 identified by immunohistochemistry and counted in the alveolar walls in order to evaluate a

147 possible correlation between AAT (native and polymerized) and the degree of lung
148 inflammation.^{25,26} Details are reported in the Online Supplement.

149 Using the semi quantitative method described by us we assessed the diseased score (inflammation,
150 muscle, wall thickness) in all airways less than 2 mm in diameter. Each bronchioles 2 mm and less
151 in diameter was examined separately for the presence of inflammatory cell infiltrate, fibrosis and
152 smooth muscle hypertrophy. For each airway, a score from 0 (normal) to 3+ (most abnormal) was
153 assigned for each pathological feature. Scores for individual features were summed and expressed
154 as percentage of maximal possible score.²⁸

155 A macroscopic quantification of emphysema was performed in all explanted lungs, using the
156 method of a Heard.²⁹ Because lungs were not fixed in inflation at a constant pressure we were not
157 able to use mean linear intercept (Lm) for the microscopic quantitation of the emphysema (air space
158 size). We did instead a semiquantitative score of the extent of microscopic emphysema (0,1,2,3+) in
159 every slide available in all cases were added and expressed as percentage of the maximal possible
160 score.²⁸

161 The possible relationship between AAT polymerization and inflammatory response was also
162 examined in liver tissue. From each liver surgical sample, two consecutive sections of 5 µm thick
163 were cut and stained with 2C1 antibody to identify polymers in one section (following the same
164 protocol used for pulmonary tissue) and with CD45 antibody to identify total leukocytes in the other
165 consecutive section. An intensity score from 0 to 3 for the extent of polymerization and of CD45
166 was graded in 50 fields for slide pair.

167 All analyses were performed using a Leica light microscope and video recorder linked to a
168 computerized image analysis system (Leica LAS w3.8).

169 **Statistical analysis**

170 Group differences were evaluated by analysis of variance (ANOVA) and unpaired Student t test for
171 clinical data, and by Kruskal–Wallis test and Mann–Whitney U test for morphological data.

172 Correlation coefficients were calculated by the Spearman rank method. P values of 0.05 or less
173 were considered to indicate statistical significance. Details are reported in the Online Data
174 Supplement.

175

176 **RESULTS**

177

178 **Clinical Characteristics**

179

180 Nine patients transplanted for severe COPD had low serum AAT levels consistent with severe
181 AATD and confirmed by either genotyping or phenotyping (8 ZZ and 1 ZI). All patients with
182 “usual” COPD, smokers without COPD and non-smokers, had a normal α_1 band on protein
183 electrophoresis.

184 The clinical characteristics of the subjects in this study are shown in Table 1. There were no
185 differences in age and amount smoked (14% current smokers and 86% recent ex-smokers). The
186 values of FEV₁ (% predicted) and FEV₁/FVC (%) were similarly decreased in the COPD with
187 AATD and in “usual” COPD, whereas they were in the normal range in smokers without COPD
188 and non-smokers.

189

190 **Histochemical and immunohistochemical findings**

191

192 Positive staining with anti-AAT antibody IR505, which stains both native and polymerized AAT,
193 was observed mainly in alveolar macrophages (AM) and occasionally in the alveolar walls (Fig.1
194 panels A-B). There was no significant difference in the percentage of alveolar macrophages positive
195 for total (native and polymerized) AAT between: COPD with AATD, “usual” COPD, smokers
196 without COPD and non-smokers (Fig.1, C).

197 The percentage of PAS positive AM was increased not only in individuals with AATD, but also in
198 smokers with or without COPD and normal AAT levels compared to non-smokers, where no PAS
199 positive intracellular inclusion were seen (Fig.2). Furthermore, the percentage of periodic acid-Schiff
200 (PAS) positive AM was also increased in smokers with “usual” COPD compared to smokers
201 without COPD. (Fig. 2) The PAS inclusions were similar to those seen in the liver from individuals
202 with PiZZ AATD (Fig.3 A-B). The use of the polymer specific 2C1 monoclonal antibody
203 (recognizing specific intracellular AAT polymers) showed a similar pattern for polymerization in
204 AM and in liver sections of PiZZ AAT individuals (Fig.3 C-D). The percentage of AM that stained
205 positive for polymers was increased not only in individuals with AATD, but also in smokers with or
206 without COPD and normal AAT levels compared to non-smokers, where no polymerization was
207 seen (Fig.4).

208 When all cases were considered together, the cumulative exposure to cigarette smoke (packs/year)
209 was positively correlated to the percentage of macrophages showing PAS+ inclusions
210 ($r=0.41;p=0.003$) and those positive for AAT polymers ($r=0.53;p=0.0001$;e-Fig 1).

211 The score of small airways disease in COPD subjects with and without AATD was significantly
212 higher than in the smokers without COPD and in non-smokers (Table 2).

213 On macroscopic analysis both transplanted groups (with and without AATD) had severe diffuse
214 emphysema with vast extension of lung destruction in both upper and lower lobes. Probably
215 because of the vast extension of lung destruction in both upper and lower lobes, the type of
216 macroscopic emphysema (CLE or PLE) was not clearly defined. The semi quantitative score of the
217 extent of microscopic emphysema showed that cases with COPD, with and without AATD, had an
218 increased emphysema score when compared with both smokers without COPD and non-smokers
219 (Table 2).

220 The number of lymphoid follicles/cm² in COPD subjects with and without AATD were
221 significantly higher than in the smokers without COPD and in non-smokers (Table 2), as did the
222 number of B and CD4+ and CD8+ lymphocytes in the alveolar wall (Table 2).

223 When we examined the relationship between the presence of polymerized AAT in alveolar
224 macrophages and the lung pathology we found that the percentage of polymerized alveolar
225 macrophages correlated significantly with the emphysema score ($r=0.55;p=0.002$), the small airway
226 disease score ($r=0.44;p=0.004$;e-Fig 2), and the numbers of neutrophils ($r=0.31;p=0.05$) and also
227 with the number of CD8+T lymphocytes ($r=0.51;p=0.002$;e-Fig 3). Furthermore, the percent of
228 polymerized AM was inversely correlated with pulmonary function parameters (FEV₁: $r=-$
229 $0.44;p=0.002$ and FEV₁/FVC: $r=-0.41;p=0.005$).

230 In liver tissue there was a positive correlation between the score of polymerization and that of
231 infiltration of inflammatory cells (CD45) ($r=0.56;p<0.0001$).

232

233 **Discussion**

234

235 Alveolar macrophages are highly prevalent within the lung and can produce considerable amounts
236 of AAT. We investigated if polymerization due to misfolding, aggregation and retention of
237 abnormal Z-AAT that takes place in liver cells, could also occur in alveolar macrophages. Our
238 results showed that AAT polymers are present in alveolar macrophages in the lung of individuals
239 with PiZZ AAT deficiency (COPD with AATD). Surprisingly, we also found AAT polymers in
240 alveolar macrophages of smokers with COPD and normal AAT levels (“usual” COPD) and in
241 smokers without COPD, but not in non-smokers.

242 The presence of significant polymerization of AAT in human alveolar macrophages directly in
243 human lung tissue had never been previously reported. Perhaps alveolar macrophage polymers are
244 the source of the bronchoalveolar lavage (BAL) polymers previously described in individuals with
245 PiZZ AAT deficiency.³⁰ We have found that periodic acid-Schiff (PAS) positive granules, possibly
246 representing protein polymers, can be seen in alveolar macrophages by light microscopy. With the
247 use of a specific antibody we showed that the PAS positive granules present in both PiZZ and
248 PiMM AAT alveolar macrophages are, at least in part, due to AAT polymerization. There was a
249 large variation in the percentage of macrophages showing AAT polymerization (ranging from 0 to
250 55%), possibly because some polymers might be too small to be detected (polymers can vary in size
251 from 2 to many molecules which can aggregate to form the visible granules).³¹ In addition, this
252 variation could also depend on the alveolar macrophages phenotype and their proportion in the
253 lung, since antiinflammatory M2 macrophages have been shown to express higher AAT mRNA,
254 and thus potentially more polymerization, than proinflammatory M1 macrophages.³²

255 **AAT contribution by alveolar macrophages.**

256 The polymerization of AAT within lung alveolar macrophages can have severe consequences for
257 lung homeostasis and the development of emphysema associated with AAT deficiency. Liver
258 produces wild-type M-AAT that diffuses through the endothelial barrier of the lung providing
259 alveolar concentration of 10-15% of the plasma AAT level,³³⁻³⁵ and this concentration would be
260 significantly supplemented by the secretion of AAT from alveolar macrophages.¹⁴ It has been
261 calculated that there are approximately 20×10^9 lung alveolar macrophages which produce three
262 times more AAT than bone marrow derived circulating monocytes,¹⁴ either because they are
263 already programmed in the fetal liver, or because they are reprogrammed by the lung micro-
264 environment promoting the more efficient and/or increased production. The fact that alveolar
265 macrophages reside directly at the site where AAT functions as an antiprotease and modulator of
266 inflammation, suggests a specific differentiation of these cells and highlights their important
267 contribution to the maintenance of lung homeostasis and its failure in deficient states.

268 **Mechanisms of AAT polymerization in the lung.**

269 It has been clearly demonstrated that under stimulation PiMM and PiZZ alveolar macrophages
270 produced similar AAT mRNA levels,¹⁴ however PiZZ alveolar macrophages produced 10 times less
271 AAT protein than PiMM alveolar macrophages. This suggests that the defect is at the secretory
272 level, and that the secretory defect secondary to protein misfolding and polymerization seen in the
273 liver, is also present in alveolar macrophages. Unexpected was the finding, never reported before,
274 of AAT polymers in the alveolar macrophages of smokers with COPD and normal AAT levels
275 (“usual” COPD) and also in smokers without COPD, but not in non-smokers. All inhibitory
276 SERPINS can be induced to polymerize by high temperature, oxidation and incubation with
277 denaturants.³¹ These agents perturb the structure of AAT, opening β -sheet A-sheet to allow
278 polymerization, although the rate of polymer formation is slower in wild-type M than mutant Z
279 AAT. It has been shown that cigarette smoke can greatly accelerate PiZ-AAT polymerization and
280 oxidize PiM-AAT in mice and human plasma³⁶ that is in keeping with the association between
281 cigarette smoking and polymerization reported in our study. This may explain our novel finding of
282 AAT polymers present in alveolar macrophages from smokers with normal levels of AAT.

283 **Possible consequences of AAT polymerization.**

284 The lung disease seen in individuals with PiZZ AAT deficiency is usually thought as secondary to
285 the low levels of circulating liver-produced AAT, to which we can now add the loss of the AAT
286 secreted by the alveolar macrophages due to AAT polymerization. Furthermore, AAT
287 polymerization could also contribute to the mechanism of disease by triggering important pro-
288 inflammatory effects. It has been previously reported that polymers of AAT in BAL from
289 individuals with PiZZ AAT deficiency³⁰ are chemotactic for human neutrophils *in vitro* and in
290 mouse models of disease.³⁷⁻³⁹ Along with a “loss of AAT function” there may be an additional
291 “toxic gain of function” originating from the accumulation of misfolded and aggregated AAT in
292 alveolar macrophages endoplasmic reticulum (ER), which could induce ‘ER stress’ and the
293 consequent, unfolded protein response (UPR) that normally ensures that misfolded proteins are
294 removed for degradation. However chronic ER stress could tip the UPR from been adaptive to
295 promoting inflammation.⁴⁰ Although we have not studied this possibility, the induction of UPR
296 secondary ER stress in blood monocytes from PiZZ AAT individuals⁴¹ and in bone marrow derived
297 macrophages⁴² has been shown to potentiate pro-inflammatory signaling, including the induction of
298 genes encoding CXC-chemokine ligand 1 (CXCL1) CXCL2, TNF, IL-1, and IL-6.⁴¹
299 Likely the following events could plausibly take place in the lungs of smokers with and without
300 AAT deficiency (Fig.5): inflammatory stimuli, cigarette smoke, free elastase, elastase-AAT-
301 complexes would stimulate an increase production of AAT in alveolar macrophages, which could

302 misfold and polymerize in the endoplasmic reticulum causing endoplasmic reticulum stress and
303 activation of the UPR. As in a vicious circle (Fig.5), UPR activation by increasing the production of
304 pro-inflammatory cytokines and chemokines, such as IL-6, would increase the inflammation that
305 will induce further AAT production, further misfolding and retention in macrophages endoplasmic
306 reticulum perpetuating the endoplasmic reticulum stress. Other local factors such as local hypoxia,
307 as seen in COPD, could add to ER stress. The correlation between the extent of polymerization and
308 the severity of inflammation in lung and liver is in support of this hypothesis. Similar mechanisms
309 are thought to play an important role in autoimmune diseases such as inflammatory bowel disease
310 and rheumatoid arthritis.⁴³⁻⁴⁵

311 If this were the case ER stress would be an important added stimulus and contributor to the innate
312 and adaptive immune inflammation that we have described in severe PiZZ AAT deficiency and in
313 “usual” COPD.²⁵ Importantly, ER stress does not always induce inflammation since cellular
314 adaptation to chronic ER stress can also suppress the inflammatory response to unfolded protein
315 (UPR).^{46,47} How cells decide between proinflammatory and anti-inflammatory UPR signaling is
316 poorly understood. This phenomenon could perhaps explain why AAT polarization is seen in our
317 population of smokers without COPD, who have less lung inflammation.

318 The findings described emphasize the complex role that could be played by the molecular
319 abnormalities of AAT in the development of COPD and emphysema and highlights another
320 important and potentially damaging effect of cigarette smoking. Our findings also highlight the
321 similarities, ever more evident, in the pathophysiology of COPD in smokers with and without AAT
322 deficiency and add another potentially important step to the complex mechanism underlying COPD.

323 **Conclusion**

324 Polymerization of AAT in alveolar macrophages occurs in the lungs of individuals with AATD but
325 also in smokers with normal AAT levels with or without COPD. Our findings highlight the
326 similarities in the pathophysiology of COPD in individuals with and without AATD, adding a
327 potentially important step to the mechanism of COPD.

328

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330 **AUTHOR'S CONTRIBUTION:**

331 Conception and design: MGC, MS, DL.

332 Performing experiments: EB, RB, CR, MT, SB, GT

333 Clinical characterization: DB, FR, SB, FC, GT, SF, AS

334 Analysis and interpretation: MGC, MS, DL, EM, EB, MPFB

335 Drafting the manuscript for important intellectual content: MGC, MS, DL, EM, EB, MPFB

336

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477 **Table1: Clinical characteristics of the subjects in the study cohort.**

| | COPD with AATD | Usual COPD | Smokers w/o COPD | Non Smokers |
|--|---------------------------|-----------------------|-----------------------------|------------------------|
| Number of subjects, n | 9 | 24 | 11 | 13 |
| Age, years | 53±3 | 57±1 | 62±2 | 56±6 |
| Smoking history, pack-years | 34±8 | 41±7 | 48±7 | - |
| Current/ex-smokers, n | 0/9 | 2/22 | 4/7 | - |
| FEV₁, % pred | 19±2 [†] | 20±2 [†] | 98±3 | 108±5 |
| FEV₁/FVC, % | 35±5 [†] | 37±3 [†] | 77±2 | 85±4 |

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479 Definition of abbreviations: AATD = α_1 -antitrypsin deficiency; COPD = chronic obstructive
 480 pulmonary disease; “usual” COPD = COPD with normal AAT levels;

481 Values are expressed as the means±SD.

482 [†] Significantly different from smokers without (w/o) COPD and non-smokers (p<0.0001).

483

Table 2: Quantification of lung pathology and inflammation.

| | COPD with AATD | Usual COPD | Smokers w/o COPD | Non Smokers |
|--|---------------------------|-----------------------|-----------------------------|------------------------|
| Small airways disease (score %) | 78(43-92)** | 67(33-100)** | 26(0-63) | 17(0-50) |
| Emphysema (score %) | 83(67-100)** | 84(33-100)** | 0(0-17) | 0(0-0) |
| Lymphoid follicles/cm ² | 4.6 (0.7-16.5)** § | 1.5(0-6.1)** | 0(0-2.5) | 0(0.0-0.8) |
| B cells/mm of alveolar wall | 2.1(0-4.4)** | 0.9(0-5.0)** | 0.2(0-0.63) | 0.3(0-0.9) |
| CD4 ⁺ cells/mm of alveolar was | 5.5(0.9-10.8)* | 6.1(1.6-11.9)* | 2.26(0.2-4) | 2.1(0-5.4) |
| CD8 ⁺ cells/mm of alveolar wall | 3.4(0.6-6.8)§ | 4.1(3.0-6.8)* | 3.4(0.6-5.1) | 2.1(0-5.2) |
| Neutrophils/mm of alveolar wall | 6.3(1.2-15.9) | 9.4(4.5-13.9) § | 6.8(2.5-9.5) | 3.8(0-15.1) |

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Definition of abbreviations: AATD = a1-antitrypsin deficiency; COPD = chronic obstructive disease; usual COPD: COPD with normal AAT levels.

Values are expressed as median(range).

*or** Significantly different from smokers without (w/o) COPD and non-smokers (*p<0.05 or **p<0.01)

§ Significantly different from usual COPD (p<0.05)

§ Significantly different from non-smokers (p<0.05)

494 **Figure Legends:**

495

496 **Figure 1. Total (native and polymerized) α 1-antitrypsin (AAT) immunostaining in alveolar**
497 **macrophages.** Quantification of AAT expression in alveolar macrophages of patients with chronic
498 obstructive pulmonary disease and α 1-antitrypsin deficiency (COPD with AATD), “usual” COPD
499 (COPD with normal AAT levels), smokers without COPD, and non-smokers.

500 (A) Representative examples of AAT expression in the lung of a COPD patient with AATD, and
501 (B) in the lung of a non-smoker. Positive staining (in brown) was mainly observed in alveolar
502 macrophages and occasionally in the alveolar wall. Immunostaining with polyclonal antibody
503 IR505 anti-AAT (A and B). Scale bars = 40 μ m.

504 (C) The percentage of alveolar macrophages positive for AAT was not significantly different
505 among the four groups of subjects examined. Horizontal bars represent median values.

506

507 **Figure 2. PAS staining in alveolar macrophages.** Quantification of PAS expression in alveolar
508 macrophages of patients with COPD and α 1-antitrypsin deficiency (COPD with AATD, “usual”
509 COPD (COPD with normal AAT levels), smokers without COPD, and non-smokers.

510 (A) Representative examples of PAS expression in the lung of a COPD patient with AATD, and (B)
511 in the lung of a non-smoker. Positive staining (in violet) was mainly observed in alveolar
512 macrophages; arrow indicate PAS positive inclusion. Scale bars = 30 μ m.

513 (C) The percentage of PAS positive alveolar macrophages was increased in patients with AATD,
514 and in smokers with and without COPD compared to non-smokers. Furthermore, the percentage of
515 alveolar macrophages positive for PAS was increased in “usual”COPD compared to smokers
516 without COPD. P values in the figure represent Mann–Whitney U tests. Kruskal–Wallis test:
517 $p < 0.0001$. Horizontal bars represent median values.

518

519 **Figure 3. PAS staining and immunostaining for AAT polymers in liver and lung sections of**
520 **AATD patients.** (A and B) Representative examples of PAS expression in the liver of a patient
521 with AATD (A) and in the lung (B) of a COPD patient with AATD. Positive PAS staining in violet.
522 (C and D) Representative examples of AAT polymers expression in the liver of a patient with
523 AATD (C) and in the lung (D) of a COPD patient with AATD. Positive immunostaining with
524 specific monoclonal antibody 2C1 specific for AAT polymers in brown (C and D). A-C: Scale bars
525 = 30 μ m. D: Scale bars = 15 μ m.

526

527 **Figure 4. α 1-antitrypsin (AAT) polymers in alveolar macrophages.** Quantification of AAT
528 polymers expression in alveolar macrophages of patients with COPD and α 1-antitrypsin deficiency

529 (COPD with AATD), “usual” COPD (COPD with normal AAT levels), smokers without COPD,
530 and non-smokers.

531 (A) Representative examples of AAT polymers expression in the lung of a COPD patient with
532 AATD and (B) in the lung of a non-smoker. Positive staining (in brown) was mainly observed in
533 alveolar macrophages; arrows indicate AAT positive polymers. Immunostaining with monoclonal
534 antibody 2C1 anti-AAT polymerized (A and B). Scale bars = 30 μ m.

535 (C) The percentage of alveolar macrophages positive for AAT polymerized was increased in COPD
536 patients with AATD, in “usual” COPD and in smokers without COPD compared to non-smokers. P
537 values in the figure represent Mann–Whitney U tests. Kruskal–Wallis test: $p < 0.0001$. Horizontal
538 bars represent median values.

539

540 **Figure 5. The pathway of lung inflammation induced by AAT polymerization.** The
541 inflammatory response induced by smoking would upregulate α -1ATmRNA in alveolar
542 macrophages. This would increase AAT production that could misfold and polymerize in the
543 Endothelial Reticulum (ER) causing ER stress that, with the enhancement of a “second hit” by
544 cigarette smoke causes activation of the Unfolded Protein Response (UPR). As in a vicious circle
545 UPR activation would further increase the expression of pro-inflammatory genes and lung
546 inflammation, which would induce further AAT production. Furthermore, the chemotactic role of
547 AAT polymers will attract neutrophils further increasing the inflammatory response, all
548 contributing to the worsening of the disease.